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14. ABSTRACT

This research project is a result of a collaboration between three research groups aimed at elucidating basic replication processes of CCHFV with the expected outcome of providing basic research reagents and establishing the foundation of knowledge necessary for discovery of vaccines and antiviral therapeutics for Crimean Congo hemorrhagic fever. Our major findings during the second year of support are the following: We have mapped domains in the N and L proteins of CCHFV responsible for protein-protein interactions and RNA-protein interactions. We have identified a novel activity associated with the N-terminal of the L protein, that is responsible for deconjugation of ubiquitin and ISG15 conjugates, that could be a target for antiviral development and for attenuation. We have detected and NSm protein produced after cleavage of the glycoprotein precursor in virus infected cells. The NSm is stable and transported to the Golgi. We have optimized the techniques to propagate CCHFV in tissue culture and generated high titer stocks. Our results provide novel insights on the molecular biology of this understudied highly pathogenic human virus.

15. SUBJECT TERMS
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1. Introduction.

This investigator-initiated project represents a collaborative team approach to the study of a highly pathogenic emerging virus of military importance, Crimean Congo hemorrhagic fever virus (CCHFV). CCHFV causes disease characterized by abrupt onset fever and can progress to hemorrhage, renal failure and shock. Mortality 13-50% is common. This severe disease is endemic in sub-Saharan Africa, the Middle East, and central Asia, all areas of current significant military operations. The extremely pathogenic nature of CCHFV has led to the fear that it might be used as an agent of bioterrorism or biowarfare. This proposal is comprised of three interrelated subprojects aimed at elucidating basic replication processes of CCHFV with the expected outcome of providing basic research reagents and establishing the foundation of knowledge necessary for discovery of vaccines and antiviral therapeutics for CCHF. The body of the progress report for each sub-project is outlined in the following pages.

2. Body.

Subproject #1: Reverse genetics of CCHFV

Subproject #1 focuses on understanding the molecular interactions between the components of the RNA replication machinery of CCHFV: the N and L proteins and the viral RNAs, with the final goal of establishing reverse genetics techniques for the rescue of CCHFV from plasmid DNA. These techniques will be used to generate attenuated strains of CCHFV. There are three tasks:

Task 1. Characterization of cis- and trans-acting signals involved in RNA replication and transcription of CCHFV.

This task included the development of expression plasmids encoding the N and L proteins of CCHFV and model vRNAs encoding reporter genes supporting the replication and transcription of this viral RNA when transfected into cells. These plasmids will be used also under this task to perform mutational analysis to identify sequence/structure regulatory motifs involved in viral RNA replication and transcription, as well as to identify domains involved in protein-protein interactions and protein-RNA interactions.

During the first year, we sequenced and cloned the L (large polymerase) and N (nucleoprotein) open reading frames of CCHFV, strain 10200 into expression plasmids, as well as generated different reporter genes (GFP and luciferase) flanked by the 3' and 5' noncoding regions of the S segment of CCHFV. In this second year of this project, we have concentrated in studying the interactions of the N protein with itself, with the L protein, and with RNA.

N-RNA Interactions. We previously showed that a GST-N recombinant protein produced and purified in *E. coli* was able to bind to ssRNA in a dot blot filter binding assay. In order to identify domains in the N protein interacting with RNA we now have made truncations in the N protein, expressed as GST fusion proteins in bacteria, and determined the ability of these truncations to bind to ssRNA in the same dot blot assay. The results are represented in Fig. 1 and indicate that the N protein has a strong RNA-binding domain in the C-terminal half of the protein (amino acids 240 to 482) and a second weaker RNA-binding domain at the N-terminus (amino acids 1 to 160). We are in the process of confirming these results using EMSA assays and we are also further mapping these two domains.

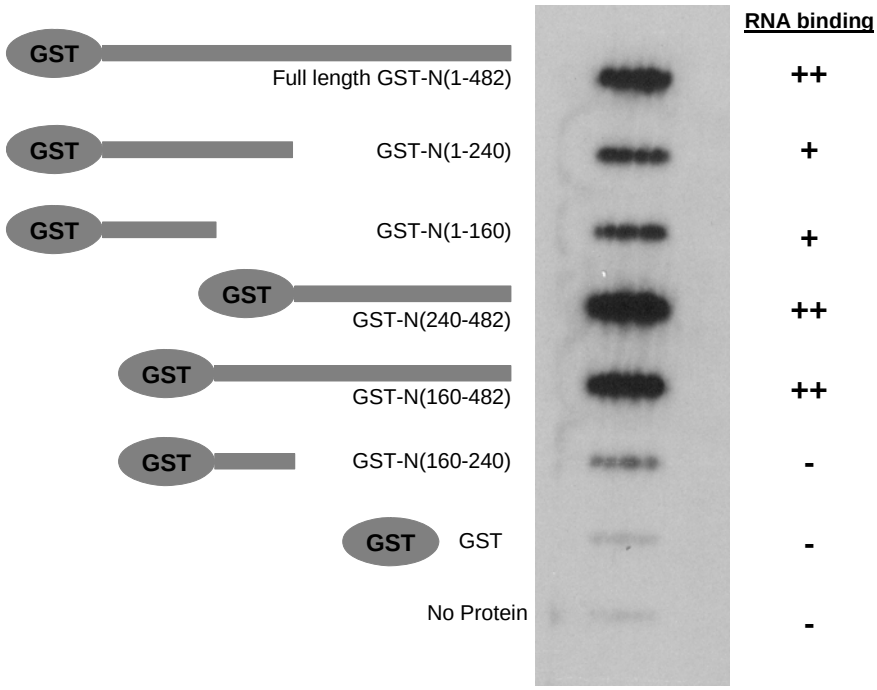


Fig. 1. RNA binding domains of the N protein of CCHFV. The depicted GST-fusion proteins were expressed in E. coli and purified using a gluthation-agarose column. Equal amount of GST-fusion proteins were GST-N or GST recombinant proteins at the indicated concentration were incubated with 12ng of radiolabeled in vitro transcribed RNA in binding buffer for 30min at 37°C and then slot-blotted onto nitrocellulose filters.

N-N interactions. Bioinformatic analysis of the sequence of the N protein of CCHFV revealed a predicted coiled-coiled region between amino acids 240 and 300 (Fig. 2). This region is likely to be involved in oligomerization of the N protein.

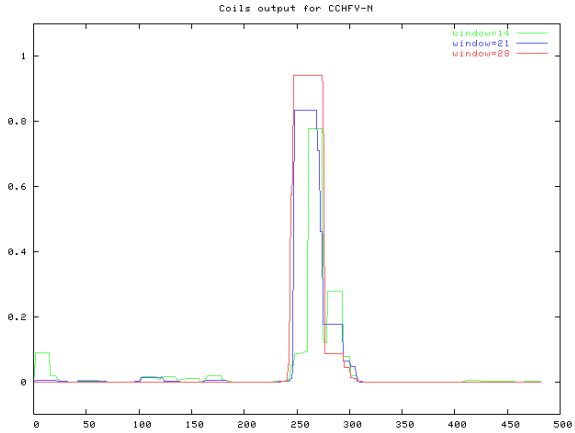


Fig. 2. Output from analysis of CCHFV-N by COILS program. The X-axis represents the 482 amino acid N sequence and the Y-axis represents the probability (with 1=100%) that an amino acid residue is within a coiled-coiled region.

In order to characterize whether the N protein is able to multimerize, we performed co-immunoprecipitations using Flag-tagged and HA-tagged N constructs expressed in 293 cells. Truncations of N similar to those shown in Fig. 1 were made to characterize the domains involved in N-N interactions. The results shown in Fig. 3 demonstrate that N oligomerizes, and that the first 160 amino acids are dispensable for N-N interaction. All N truncations lacking the predicted coiled-coiled domain were unable to interact. We are currently fine mapping the area required for oligomerization of N.

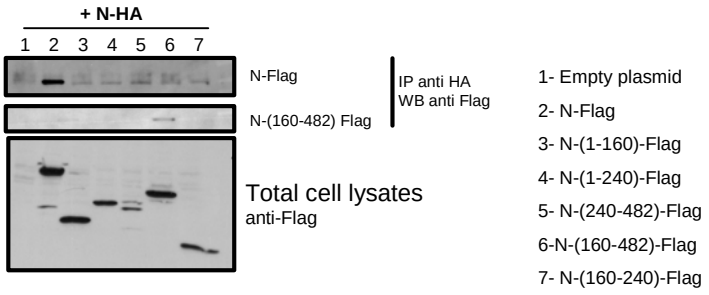


Fig. 3. N-N interactions. A plasmid expressing HA tagged N (N-HA) was cotransfected with empty plasmid or with plasmids expressing Flag tagged full length N or truncated N mutants. Two days after transfection, cell extracts were made, immunoprecipitated with anti-HA antibody, subjected to SDS-PAGE, and western blotted with anti-flag antibodies.

N-L interactions. We also investigated the potential ability of the N protein to interact with the L protein. For this purpose, we divided the L protein in three fragments of approximately 3 kbases each, and expressed as HA-tagged constructs together with N-Flag. Co-immunoprecipitations revealed a strong interaction of N with the N-terminal fragment of L, and a weak interaction with the C-terminal fragment. No interaction was observed with the middle portion of L (Fig. 4). We are in the process of fine mapping these two interaction domains present in L.

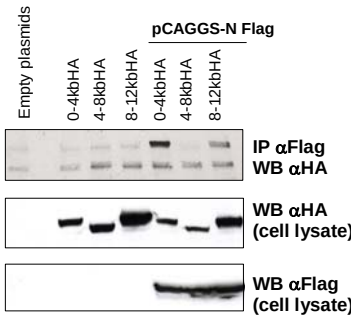


Fig. 4. N-L interactions. A plasmid expressing Flag tagged N (N-Flag) was cotransfected with plasmids expressing HA tagged L fragments. Two days after transfection, cell extracts were made, immunoprecipitated with anti-Flag antibody, subjected to SDS-PAGE, and western blotted with anti-HA antibodies.

Other functions of the CCHFV replication components. In our original application we have proposed to develop mini-genome systems to study the replication and transcription of CCHFV RNAs. While we have successfully cloned the L and N proteins into mammalian expression plasmids, we have not yet been able to demonstrate significant transcription and replication of minigenomes consisting of luciferase and GFP reporter genes flanked by the noncoding regions of CCHFV RNAs. There are several reasons that might be responsible for this. These include levels of expression of the different components (N, L and minigenome), need of coding portions of the CCHFV genes for replication and transcription, and point mutations in the N and/or L plasmids resulting in non-functional proteins. We are currently exploring these possibilities, and therefore, we have moved the development of replicative minigenomes as well as tasks 2 and 3 (necessitating of transcriptionally active L and N proteins) to years 3 and 4 of the proposal. However, while conducting these experiments, we and others reported the presence of an OTU-like domain at the N-terminus of the L protein of CCHFV. Recent studies have attributed to the Ovarian Tumor (OTU) superfamily of cysteine proteases a role as putative deubiquitinating (DUB) enzymes (2, 5). We therefore have characterized the CCHFV-L OTU domain in relation to its potential isopeptidase activity towards Ub- and ISG15-conjugated proteins. Expression of CCHFV-L resulted in deISGylation of cellular proteins. This activity was mapped to its OTU domain. Moreover, L truncated proteins expressing the catalytic core OTU domain also have deubiquitinating activity, both in vitro and in cell-based transfection experiments. Mutational

analysis indicated that Cys40 is fundamental for the OTU domain catalytic activity. As protein ISGylation has an anti-viral effect, the observed deISGylation activity of the full length CCHFV-L protein suggests a novel viral strategy to circumvent the host cell antiviral machinery to its benefit. The identification of this isopeptidase activity may permit the screening of specific viral inhibitors for CCHFV and other OTU-domain encoding viruses. We are currently conducting experiments in collaboration with subproject 3 to demonstrate deISGylation and deubiquitination activities in CCHFV-infected cells.

Task 2. Generation of recombinant CCHFV from cDNA. We are still conducting experiments towards the expression of transcriptionally functional N and L proteins. Once we achieved this, we will develop this task under BSL-4 containment in collaboration with subproject 3.

Task 3. Generation of attenuated CCHFV by reverse genetics. As with task 2, this task will be developed in the next years of this proposal. Importantly, the identification of de-ubiquitination and deISGylation activities associated with the OTU domain of L (see above) points to this activity as a unique and novel target for viral attenuation.

Subproject #2: Cell Biology of CCHFV Glycoproteins

Work carried out under subproject #2 is being performed in the laboratory of Dr. Robert W. Doms at the University of Pennsylvania, and is directed towards characterizing the envelope glycoproteins of CCHFV. There are two tasks:

> *Task 1*. Analyze the assembly and processing of CCHFV G1 and G2 glycoproteins and characterize the mucin-like domain present at the amino terminus of the polyprotein. (Years 1-3)
>
> *Task 2*. Identify cell receptors and attachment factors for CCHFV.

Over the two years of this DOD-sponsored project, two papers have been published, one in the Journal of Virology and a second in Journal of General Virology (1, 3). These were included in our last previous report. A third paper that addresses *Task1* will be submitted in the fall of 2007. Both published studies were done in collaboration with our colleagues at USAMRIID and Mt. Sinai, and the third paper has been done in part at USAMRIID. Mr. Lou Altamura, the graduate student who leads this project, visits USAMRIID approximately 4 times a year, and spends 1-2 weeks each time to work with live CCHFV in the biocontainment suites.

Task 1. Analyze the assembly and processing of CCHFV G1 and G2 glycoproteins and characterize the mucin-like domain present at the amino terminus of the polyprotein.

In year 1, as described in our previous report, much of our effort was spent on developing antibodies and protein expression systems. In year 2, we have made considerable progress in studying the processing of the viral glycoproteins, G_N (formerly G1) and G_C (formerly G2) especially that of Gn. The CCHFV glycoproteins are derived from the polyprotein precursor encoded by its medium (M) RNA segment through a series of endoproteolytic cleavage events. The M polyprotein is first divided into two intermediate precursors, $PreG_N$ and $PreG_C$. The $PreG_N$ precursor is subject to extensive proteolytic processing, with the N-terminal mucin-like and GP38 domains being removed by furin and SKI-1 mediated proteolysis, respectively. SKI-1 cleavage generates the N-terminus of mature G_N at amino acid 520 (in strain IbAr10200), and a presumably related protease cleaves the N-terminus of $PreG_C$ to yield a mature G_C beginning at amino acid 1041. There are four predicted transmembrane helices between the G_N and G_C

ectodomains, which define two relatively large (approximately 100 amino acids each) cytoplasmic loops and a short (15 amino acids) lumenal domain contained within them. If G_N were defined as amino acids 520-1040, then this protein would have a predicted mass of 58 kDa, excluding posttranslational modifications such as glycosylation. However, G_N migrates with an approximate molecular weight of 37 kDa in SDS polyacrylamide gels, suggesting that additional cleavage event(s) in the topologically complex region between $PreG_N$ and $PreG_C$ must occur.

To test this hypothesis, a plasmid ($PreG_NV5(961)$) was constructed that encoded amino acids 1-961 of the IbAr10200 M polyprotein and a C-terminal vector-derived V5-His_6 cassette . This version of $PreG_N$, which included three of the four predicted transmembrane helices following the G_N ectodomain and most of the second cytoplasmic loop, was designed based upon a previous report which operationally defined $PreG_C$ as amino acids 962-1684 of the polyprotein. When lysate of 293T/17 cells expressing $PreG_NV5(961)$ was analyzed by SDS-PAGE followed by immunoblotting with a polyclonal antisera specific to the G_N ectodomain, $PreG_N$ (140 kDa) and G_N (37 kDa) species were observed. Furthermore, these bands were of comparable molecular weights to those generated from a full-length M segment possessing a V5-His_6 cassette at the C-terminus of G_C ($M[G_CV5]$), thereby indicating that G_N was processed similarly in these two contexts.

When the blot was probed with an antibody specific to the C-terminal V5 epitope, $PreG_N$ was also detected in the $PreG_NV5(961)$ lysate. In the lysate of $M[G_CV5]$, G_C was detected, which is in agreement with different placement of the V5 epitope between the two constructs. The mature G_N glycoprotein, however, was not detected in the $PreG_NV5(961)$ lysate. Instead, a band with a molecular weight of approximately 20 kDa was detected. This presence of this V5-tagged fragment indicated that a cleavage event not only occurred somewhere within the C-terminal region of $PreG_N$, but that this event occurred efficiently, and prior to the N-terminal cleavage events that liberate the mucin-like and GP38 glycoproteins from G_N. If this C-terminal cleavage event occurred later in the biosynthetic pathway, then one would have expected to see additional larger processing intermediates containing the G_N ectodomain and perhaps also the GP38 domain. To determine if these cleavage events might be specific to 293T/17 cells, $PreG_NV5(961)$ was expressed in additional cell lines known to support CCHFV replication (BHK, CHO-K1, HeLa, Huh-7, Vero E6, and SW-13), and it was found that C-terminal cleavage of $PreG_N$ occurred efficiently in all cell lines tested (data not shown). Thus, it appeared that addition of an epitope cassette allowed for the identification of a novel, C-terminal proteolytic fragment of $PreG_N$.

We have done additional work to characterize the C-terminal fragment, that in many ways resembles an NSm protein. We that that this protein is produced efficiently and shortly after synthesis of PreGn, that it is stable, and that at least some of it is transported to the Golgi. We have managed to make a rabbit antisera to this protein that reacts by western blot. Using this, we have found that this protein is produced upon expression of M segments from diverse CCHFV strains. Importantly, Lou Altamura, the student who has done this work, visits USAMRIID several times a year, and has been trained to work in the biocontainment suites. He has found that this NSm protein is also produced in cells infected with the 10200 strain of CCHFV. Thus, this protein is not an artifact of transient expression. To determine the function of this protein, we really need a reverse genetics system. In the absence of this, we will express this protein and determine if it interacts with Gn or Gc.

We have also begun our work on the mucin domain in earnest. We do not have much to report at present, but we are in the midst of producing a relatively large number of recombinant vaccinia virus and baculovirus vectors that express different portions of Gn, including the mucin domain by itself, mucin and gp38, and the Gn ectodomain. The mucin-gp38 protein is secreted from cells, and we have been able to purify it. The goals of these studies are to produce soluble proteins, including the mucin domain, in quantity for cell binding and for crystallization studies.

Task 2. Identify cell receptors and attachment factors for CCHFV.

We have not made much progress on this Task in year 2, as we have concentrated on Task 1. We have actually had better luck with Rift Valley Fever virus, and while not directly relevant to Task 2, we hope that the approaches we have developed for Rift Valley Fever virus will also work for CCHFV. We have developed a cell-cell fusion assay for CCHFV that we described last year, but we are hoping to develop a virus pseudotype system. We have been able to express RVFV glycoproteins and pseudotype them onto VSV virus, and in Year 3 we will try this with CCHFV, using the codon-optimized construct we produced in Year 1.

Subproject #3: Characterization of CCHFV strains and small animal model development

Work carried out under subproject #3 is being performed in the laboratory of Dr. Connie Schmaljohn at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID), and is directed at working with the intact virus in high biocontainment and supporting subprojects 1 and 2 with reagents and experiments with virus to complement in vitro work from subproject 1 and 2. The precise tasks are listed below:

Task 1. Determination of genetic factors of virulence by comparison of tick, livestock and human isolates of CCHFV.

a. <u>Sequence analysis</u>

During this reporting period we established collaboration with investigators at Lawrence Livermore National Laboratories (LLNL) for complete sequence analysis of CCHFV strains. To accomplish the goal of genetically characterizing CCHFV from primary human and tick isolates, we propagated 5 Uzbekistan strains of CCHFV from human sera (H-35, I-40, I-248, I-313, P20), and 1 strain from ticks (KKK28) at BSL4 conditions and provided the RNA for sequencing to LLNL. Currently, 3 of the strains have been PCR-amplified and are undergoing analysis.

In addition to our own studies, investigators at the Centers for Disease Control also are working on the genetic characterization of CCHFV. They recently reported the complete genome sequences of 13 strains of CCHFV, which were collected from diverse geographical areas (4). They found S, M and L nucleotide and amino acid sequences to be highly variable, although geographic lineages were observed. These studies suggest that CCHFV is very mutable, thus differs from most other arthropod borne viruses. These findings impart even more importance to the need to adequately characterize the antigenic and genetic diversity among primary isolates for our studies.

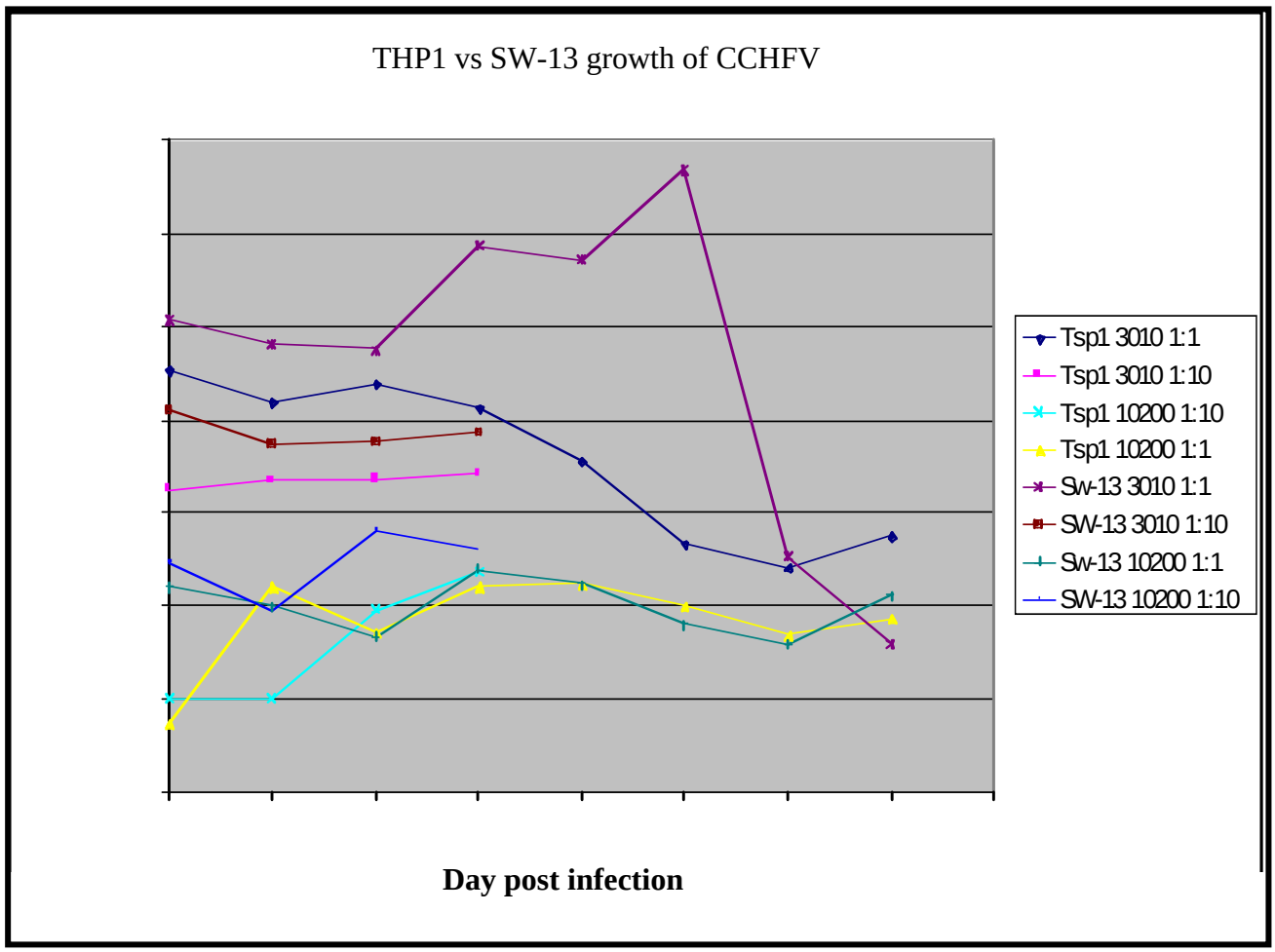
b. <u>Perform viral growth curves, cytokine analysis and preliminary gene expression profiling.</u>

1) During this reporting period, we performed studies aimed at determining an optimal cell line for virus growth. Earlier studies indicated that that HepG2 cells produce the greatest number of progeny. Currently growth curves were analyzed for CCHFV strains 10200 and 3010 in SW13 (human adrenal cortex line) cells and THP-1 (human monocyte line) cells using a quantitative real-time PCR assay that we developed. As shown in Figure 5, we found that SW013 3010 cells produced the highest levels of virus at day 5, released into the cell culture medium. However, the number of genomes detected, was far higher than actual

levels of infectious virus, as detected by plaque assay. Consequently, additional studies were undertaken to develop better methods for recovering infectious virus. This effort is reported below as part of task 3 progress.

In addition to these viral growth studies, we also have used techniques such as IFA, quantitative real-time PCR, and western blotting to show that confirmed that CCHFV replicates within the following cell lines:, 293T, HeLa, Vero E6, Huh-7. Also we have begun to optimize parameters for immuno-electron microscopic and confocal microscopic visualization of CCHFV inside infected cells. We anticipate that we will have data to report for these efforts shortly.

Figure 5. SW-13 or TSP1 cells were infected with CCHFV strains 3010 or 10200 and supernatants were collected at days 0-7 after infection. Viral genomes were detected RT-PCR.



2) To began an assessment of virulence factors for CCHFV we have been attempting to identify and characterize an interferon antagonistic response. To date, the IFN antagonism has been reported for two other hemorrhagic fever viruses, Ebola virus and Rift Valley fever virus. One of the most common methods of IFN antagonism by RNA virus is interference with the JAK-STAT signaling pathway. To determine if CCHFV was inhibiting this pathway, we infected (MOI of 5) or

mock-infected 293T cells. On days 2 and 3 after infection, cells were either treated with 1000 IU of IFN-α/ml for 30 min or left untreated. At appropriate times, cells were lysed and lysates harvested and analyzed by western blotting to measure phosphorylation and activation of STAT1 and STAT2. We found that CCHFV did not inhibit phosphorylation of STAT1 and STAT2 following IFN treatment when compared to mock infected. Also, in infected cells that did not receive any IFN treatment, there were detectable levels of phosphorylated STATs indicating and viral infection alone was inducing IFN production.

In addition, in collaboration with Dr. García-Sastre's laboratory (subproject 1), we performed studies to determine if interferon stimulated gene 15, a ubiquitin homologue is inhibited by infection with CCHFV. This experiment was undertaken because of information Dr. García-Sastre obtained during his studies to develop a reverse genetics system for CCHFV. In those studies, he found that the L segment of CCHFV contains an OTU domain (ovarian tumor superfamily of proteins), which could be associated with control of the activity of one IFN inducible gene, ISG15. To address the potential importance of this domain in ISG15 function during CCHFV infections, we transfected 293T cells with plasmids expressing ISG15, E1, and E2. 24h post-transfection, the cells were mock-infected or infected at an MOI of 5 with CCHFV strain 10200. Two days post-infection, lysates were harvested and western blots were performed to look at levels of ISG15 conjugated proteins. No differences were observed between and mock-infected and CCHFV-infected cells. However, the levels of viral infection achieved under these conditions were not optimal. Experiments are continuing using other conditions of transfection and infection.

Task 2: Epitope mapping of CCHFV monoclonal antibodies

Although we have not yet begun studies to map specific epitopes using our library of MAbs, we have provided the antibodies in support of Dr. Doms lab for studies on the antigenic characterization of CCHFV strains. Two peer reviewed reports of those studies were published last year (1, 3).

To map the epitopes, we plan to obtain a peptide library representing M segment-encoded proteins from CCHFV, strain 10200. We will use peptide ELISA to map linear epitopes recognized by the MAbs. This approach was successfully employed for Ebola virus in other studies, resulting in identification of novel antigenic determinants on the viral glycoproteins.

Task 3. Develop a mouse model for CCHFV.

A mouse protocol was approved by the Animal use committee at USAMRIID, and an attempt to develop a mouse model was conducted as follows (excerpted from animal protocol):

Approach: The goal of this protocol is to develop a mouse-adapted CCHF virus strain that is lethal in both male and female BALB/c mice at age 28 days. The method to achieve this goal is by serial passage of virus in young BALB/c mice using subsequent passages in older mice to obtain a virus that is lethal in adult mice. Virus is passaged by inoculating 5 litters of 7-day old

BALB/c mice subcutaneously (s.c.) or intraperitoneally (i.p.) with 100 pfu of virus stock. Litters are examined on a daily basis for weight change and death.

The procedure for virus passage and adaptation in 12 day old mice is:

- Starting at age 12 days and each subsequent day, one entire litter of mice is sacrificed when the litter experiences a mortality of 20% until all 5 litters have been sacrificed. Sacrificed mice are euthanized according to protocol and exsanguinated. Livers are removed and homogenized in growth medium with the clarified supernatant collected, aliquoted and frozen.
- The next virus passage is initiated by using clarified material from the 14-day-old mice liter that was sacrificed to inoculate 5 new litters of 7-day-old mice, at a dose of 0.02 ml s.c. or i.p./mouse. In the event that most or all mice die from virus infection prior to age 14 days, frozen material from the earlier time point are used to inoculate the next round of litters.
- This procedure is repeated in 7-day-old mice until the mean time to death has decreased by 3 days or more, giving evidence of virus adaptation, or until 7 total passages have been performed.

As susceptibility to infection and virus adaptation is dependant on the age of the mouse, complete virus adaptation requires serial passage in older populations of mice.

The procedure for virus adaptation to older mice is:

- Material harvested from the last 7-day-old litter in the serial passage noted above, is used to inoculate 5 litters of 14 day-old suckling mice and one litter of 7-day-old mice (7-day old mice are required as controls to assess virulence of the stock). Starting at age 19 days and each subsequent day, one entire litter of mice will be sacrificed when the litter experiences a mortality of 20% until all 5 litters have been sacrificed. Sacrificed mice will be euthanized according to LACUC protocol and exsanguinated. Livers will be removed and homogenized in growth medium with the clarified supernatant collected, aliquoted and frozen.
- The next virus passage is initiated by using clarified material from the 21-day-old mice liter that was sacrificed to inoculate 5 new litters of 14-day-old mice, at a dose of 0.02 ml s.c. or i.p./mouse. In the event that most or all mice die from virus infection prior to age 21 days, frozen material from the earlier time point is used to inoculate the next round of litters.
- This process will be repeated until a virus is obtained that is 80% lethal for 28-day-old weanling female and male mice.

This series of experiments did not yield a virus capable of killing adult mice. The experiments could not be continued due to problems associated with the BSL4 suite renovation. Consequently, during the next reporting period, we will repeat this study, and possibly carry it out further. In addition, we have submitted another mouse protocol, using a different approach to adaptation. Experiments to be conducted (excerpted from the protocol) follow:

<u>Develop mouse-adapted M CCHFV using serial passage of field isolated, low tissue culture passed virus in SCID mice followed by passage in adult mice.</u>

Approach*:* The general approach will be based on virus passage in SCID mice followed by passage in adult BALB/c mice to develop a lethal mouse-adapted CCHFV. In this portion of the adaptation protocol the goal is to isolate the virus population that is capable of migrating to target

tissues/organs (i.e. liver, peritoneal monocytes, Kupffer cells) at the earliest time point. The isolated virus will then be used to infect new mice. Each group will consist of 10 mice that will be inoculated intraperitoneally with 1000 pfu of CCHFV. Two mice will be sacrificed each on days 2 and 3 and the tissues removed and virus isolated by homogenizing the tissues in 10 mls of PBS. To isolate virus, liver homogenates will be centrifuged and the supernatants containing virus will be collected. The supernatants will be used for virus titration on Vero or other cells where appropriate to determine the viral titers. Lethality will be monitored in the remaining 8 mice. If the virus is not lethal, the liver samples with the earliest isolation of virus will be used to challenge an additional 10 mice in the same manner.

Task 4. Support using live virus for projects 1 and 2.

Several support functions have already been detailed in tasks above. In addition to those, the following support tasks were accomplished in this reporting period. Optimization of virus production:

Support of Task 1, subproject 2 (Dr. Doms Laboratory): The following studies were conducted by Dr. Doms graduate student, Lou Altamura in the BSL3 laboratories of USAMRIID:

1) In the past, we have been unable to produce high-titer stocks of CCHFV for use in our various assays. Typically, our titers were only in the range of 10^3-10^5 pfu/ml. In many cases, this meant that our experiments were limited to using at most a multiplicity of infection of 0.1-1. Recently, we began using a new freeze-thaw method, which allows us to liberate virions retained intracellularly. Using this approach, we have obtained CCHFV titers in the range of 10^7-10^8 pfu/ml, thereby allowing us to infect cells at much higher MOI values and to generate more robust signals from our data. Currently, we have only applied this technique to the IbAr10200 prototype strain. In the future, we hope to apply this method to the production of high-titer stocks of primary isolates of CCHFV in the hopes to performing more comparative studies across multiple strains.
2) We generated an ELISA antigen by PEG precipitation of CCHFV, strain 10200, released into cell culture medium. This antigen was tested and shown to be reactive with mouse hyperimmune mouse ascitic fluid generated to CCHFV.

Support of subproject 1 (Dr. García-Sastre's Laboratory):

1) Numerous unsuccessful attempts were made at optimizing and utilizing the reverse genetics system for CCHFV minigenome luciferase reporter assay. These tests were performed using Vero, 293T, and SW13 cell lines with CCHFV strains 10200 and U40. The information obtained during optimization of minigenome assays conducted under task 1, subproject 1, using supportive plasmids will hopefully generate the information required for the use of live CCHFV to drive higher levels of reporter gene expression.
2) As already mentioned, we are collaborating with subproject 1 to assay the ability of CCHFV infection to mediate deISGylation and possibly de-ubiquitination of cellular proteins involved in the antiviral response.

3. Key research accomplishments.

Our key research accomplishments are listed under **Conclusions** (Section 5) and in the three papers and two abstract listed under **Reportable Outcomes** (Section 4).

4. Reportable outcomes.

Two papers were submitted since the beginning of this project, with both papers being accepted for publication. During the second year of this proposal, one paper is almost ready to be submitted, and three abstracts have been presented in scientific meetings. These are listed below.

PAPERS

Bertolotti-Ciarlet, A., Smith J., Strecker K., Paragas J., Altamura L., McFalls J., Frias-Staheli N., García-Sastre, A., Schmaljohn, C., and Doms, RW. (2005) Cellular localization and antigenic characterization of Crimean-Congo hemorrhagic fever virus glycoproteins. J.Virol. 79: 6152-61.

Ahmed, A., McFalls, J., Hoffmann, C., Filone, C.M., Stewart S. M., Paragas, J., Khodjaev S., Shermukhamedova D., Schmaljohn, C.S., Doms, R.W., and Bertolotti-Ciarlet, A. (2005) Presence of broadly reactive and group-specific neutralizing epitopes on newly described isolates of Crimean-Congo hemorrhagic fever virus. Journal of General Virology. 86: 3327-36.

Altamura, L.A., A. Andrea Bertolotti-Ciarlet, J. Teigler, J.J. Paragas, C.S. Schmaljohn and R.W. Doms. Identification of a novel C-terminal cleavage of Crimean-Congo hemorrhagic fever virus PreGN that leads to generation of an NSM protein. (almost ready to be submitted)

ABSTRACTS TO MEETINGS (2006 only)

Altamura, L.A., Bertolotti-Ciarlet, A., Teigler, J., Paragas, J., Schmaljohn, C., and Doms, R.W. (2006) Identification of a novel C-terminal cleavage of Crimean-Congo hemorrhagic fever virus PreGN. American Society for Virology 25th Annual Meeting. July 15-19 Madison, WI.

Altamura, L.A., Bertolotti-Ciarlet, A., Teigler, J., Paragas, J., Schmaljohn, C., and Doms, R.W. (2006) Identification of a novel C-terminal cleavage of Crimean-Congo hemorrhagic fever virus PreGN. XIII International Conference on Negative Strand Viruses. June 17-22 Salamanca, Spain.

Frias-Staheli, N., Ratnakumar, K., and García-Sastre, A. (2006). The nucleocapsid of Crimean-Congo hemorrhagic fever virus is a ssRNA-binding protein that oligomerizes and can interact with the viral polymerase. XIII International Conference on Negative Strand Viruses. June 17-22 Salamanca, Spain.

5. Conclusions.

The major conclusions reached during the second year of this project are as follows:

A. The N protein of CCHFV has a strong RNA-binding domain in the C-terminal half of the protein (amino acids 240 to 482) and a second weaker RNA-binding domain at the N-terminus (amino acids 1 to 160).

B. The N protein has a predicted coiled-coiled domain that might be involved in oilgomerization.

C. The N protein oligomerizes, and the first 160 amino acids are dispensable for N-N interactions.
D. The N protein has a strong interaction with the N-terminal fragment of the L protein, and a weak interaction with the C-terminal fragment of the L protein.
E. The OTU-like domain of the L protein has isopeptidase activity and is able to mediate de-ubiquitination and deISGylation of cellular proteins
F. The Gn protein undergoes a C-terminal cleavage event that liberates an NSm protein.
G. The NSm protein is stable, transported to the Golgi, and is produced in virus infected cells.
H. It is possible to produce some soluble versions of the Gn protein that are secreted from cells, making it possible to begin crystallization trials.
I. We have established a collaboration with investigators at Lawrence Livermore National Laboratories (LLNL) for complete sequence analysis of CCHFV strains.
J. CCHFV infection does not inhibit phosphorylation of STAT1 and STAT2 following IFN treatment.
K. CCHFV infection resulted in IFN production, as detected by STAT phosphorylation.
L. We have optimized the tissue culture techniques to propagate strain 10200 of CCHFV and obtained CCHFV titers in the range of 10^7-10^8 pfu/ml.

6. References.

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3. **Bertolotti-Ciarlet, A., J. Smith, K. Strecker, J. Paragas, L. A. Altamura, J. M. McFalls, N. Frias-Staheli, A. García-Sastre, C. S. Schmaljohn, and R. W. Doms.** 2005. Cellular localization and antigenic characterization of crimean-congo hemorrhagic fever virus glycoproteins. J. Virol. **79:**6152-6161.
4. **Deyde, V. M., M. L. Khristova, P. E. Rollin, T. G. Ksiazek, and S. T. Nichol.** 2006. Crimean-Congo hemorrhagic fever virus genomics and global diversity. J. Virol. **80:**8834-8842.
5. **Evans, P. C., T. S. Smith, M. J. Lai, M. G. Williams, D. F. Burke, K. Heyninck, M. M. Kreike, R. Beyaert, T. L. Blundell, and P. J. Kilshaw.** 2003. A novel type of deubiquitinating enzyme. J. Biol. Chem. **278:**23180-23186.

7. Appendices.

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